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A *Babesia bovis* gene syntenic to *Theileria parva p67* is expressed in blood and tick stage parasites☆

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ABSTRACT

Completion of the *Babesia bovis* (T2Bo strain) genome provides detailed data concerning the predicted proteome of this parasite, and allows for a bioinformatics approach to gene discovery. Comparative genomics of the hemoprotozoan parasites *B. bovis* and *Theileria parva* revealed a highly conserved syntenic block of genes flanking the *p67* gene of *T. parva,* a sporozoite stage-specific vaccine candidate against East Coast fever (ECF). The syntenic gene in *B. bovis*, designated *bov57*, encodes a protein of limited amino acid sequence identity (11.8%) to *p67*. Monoclonal antibodies were produced against recombinant BOV57 and were used to demonstrate expression of BOV57 in merozoite and kinete stages of the T2Bo strain of *B. bovis*. Transcript levels of *bov57* in kinetes were increased 100-fold in comparison to *msa-1*, a previously identified gene encoding an erythrocyte stage surface protein. Amino acid sequence comparisons between the T2Bo strain and two attenuated and virulent strains from Argentina and Australia revealed a high degree of sequence conservation in BOV57 among these geographically and pathogenically divergent isolates (97% amino acid sequence identity). Additional genomic comparisons show that the *bov57* gene locus is also conserved in *Babesia bigemina* and *Babesia equi*. While not identifiable through amino acid or nucleotide sequence similarity, the conserved gene order within this locus in multiple piroplasms may suggest a critical function adapted for each species' unique host and life-cycle.
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1. Introduction

Arthropod transmitted apicomplexan protozoa are a serious threat to animal health and the economic stability of livestock industries worldwide. Members of the genus *Babesia* cause piroplasmosis in bovine (*B. bovis* and *B. bigemina*) and equine (*B. equi* and *B. caballi*) hosts (Purnell, 1981). Infective *B. bovis* sporozoites invade bovine host erythrocytes following transmission by the tick vectors *Rhipicephalus* (*Boophilus*) *microplus* and *R. annulatus*. Merozoites replicate within host erythrocytes and are acquired by adult female *Rhipicephalus* that transovarially transmit the kinete stage to larval progeny. *Rhipicephalus* spp. ticks were eradicated from the United States and confined to a permanent quarantine zone along the U.S border with Mexico. However, with emerging acaracide resistance, dissemination of these vector ticks by wildlife hosts, and the existence of subclinical persistently infected cattle, there is an increased risk that bovine babesiosis will re-emerge

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in the United States. The economic significance of this risk directs the need to identify novel methods to prevent tick-borne transmission and resulting disease. Comparative genomics provides a means to discover new genes and gene families potentially leading to novel interventions of parasite life-cycles in their vector and mammalian hosts.

Annotation of several related apicomplexan genomes provides the opportunity to compare the predicted proteomes of these pathogens. Orthologous genes are typically identified based on shared sequence identity in related organisms. However, sequence divergence may result in limited amino acid sequence identity between genes with similar functions or ancestry. The term synteny refers to the conservation of gene order in loci of related species. Syntenic genes that are surrounded by identical genes in distinct genomes are more likely to be true ancestral copies. Identification of homologous genomic regions between species facilitates annotation and provides evidence of predicted gene function. Thus, the identification of homologs and conservation of gene order are intertwined (Campbell et al., 2007; Rodelsperger and Dieterich, 2008). Analysis of syntenic regions in related apicomplexa is an additional approach used to locate related genes and potential targets for intervening in the pathogen life-cycle through transmission blocking vaccines.

Infections with *Theileria parva*, the causative agent of ECF, and *Theileria annulata*, which causes tropical theileriosis, have negatively impacted cattle production in tropical and subtropical regions of the world. Early studies investigating protective immunity against *T. parva* implicated sporozoite stage proteins as major targets of protective neutralizing antibodies. The p67 protein, found exclusively on the sporozoite stage, was identified as a specific target of neutralizing IgG2 antibodies (Musoke et al., 1982) and is highly conserved (100% identity) among cattle-derived parasite stocks (Nene et al., 1996). SPAG-1, a vaccine candidate against *T. annulata* and homolog of p67, has 47% sequence identity with *p67* and contains cross-reactive sporozoite neutralizing epitopes (Knight et al., 1996; Musoke et al., 1984; Hall et al., 2000). Both proteins have been a major focus of vaccine trials in cattle. Immunization with full length recombinant p67 or just the C-terminal region expressed in *Escherichia coli* resulted in reduction of severe ECF in experimentally challenged cattle (Bishop et al., 2003; Musoke et al., 2005). Although not definitively demonstrated, the proposed functions of p67 and SPAG-1 revolve around their suspected involvement in host cell recognition and/or invasion (Boulter et al., 1994; Shaw et al., 1991).

The *T. parva* genome was completed in 2005 (Gardner et al., 2005). Following the more recent completion of the virulent T2Bo strain of *Babesia bovis*, extensive chromosomal synteny between *B. bovis* and *T. parva* was identified (Brayton et al., 2007). Of particular interest was a region of synteny with *B. bovis* at the *p67* and SPAG-1 loci, both highly studied vaccine candidates against ECF and tropical theileriosis, respectively. Based on synteny, we identified loci in *B. bovis*, *B. equi*, and *B. bigemina* corresponding to the p67 locus of *T. parva*. There are six syntenic genes in this locus. While gene order is conserved between *Babesia* sp. and *T. parva*, sequence identity between p67 and the corresponding gene is lacking. We characterized the syntenic gene to p67 in *B. bovis*, which we have designated *bov57*, in the tick transmissible strain T2Bo by (1) testing for the presence of transcripts in tick and cultured blood stages, as well as verifying transcript length as predicted by the genome; (2) comparing transcript levels to a known surface protein encoding gene; and (3) verifying expression in cultured blood stages using monoclonal antibodies developed against the recombinant protein. In addition, amino acid sequence comparisons of syntenic genes in other *B. bovis* strains as well as *B. equi* and *B. bigemina* were performed to evaluate the level of sequence conservation within *B. bovis* strains and between other *Babesia* species.

2. Materials and methods

2.1. Transcript characterization

The presence of transcripts in different life-cycle stages was determined by reverse transcriptase-PCR (rt-PCR). Total RNA was isolated from erythrocytic merozoite cultures of the T2Bo strain of *B. bovis* or hemolymph from *R. microplus* females harboring >10 kinetes per high powered field as quantified by light microscopy (Howell et al., 2007a). To obtain RNA from merozoites, erythrocytes containing merozoites (30% parasitemia) were pelleted by centrifugation. Briefly, 100 μl aliquots of pellet were added to 500 μl of Trizol (Invitrogen, Carlsbad, California) and RNA isolated using standard protocols for cells grown in suspension. RNA was isolated from 100 μl infected hemolymph frozen in 500 μl Trizol using the same protocol. Following DNase treatment, rt-PCR was performed using forward primer 5′-GCGCTGAAGGCACTAGAGG-3′ and reverse primer 5′-AGGTTCGGTTGGATACGGA-3′ with a SuperScript One-Step rt-PCR kit (Invitrogen). A SMART RACE cDNA Amplification kit (Clontech, Mountain View, California) was used to generate RACE cDNA. Primer sequences used for T2Bo strain hemolymph 3′ RACE hemolymph were: forward primer 5′-ATGGCATTTGCAAAGTTGTC TATT-3′; SMART RACE nested universal primer 5′-AAGCAGTGGTATCAACGCAGAGT-3′. For infected erythrocyte 5′ RACE, previously described (Suarez et al., 2006) RACE cDNA was analyzed using the reverse primer 5′-AGCTATGGGAGTGGTATCAGTTAT-3′ and the SMART RACE nested universal primer 5′-AAGCAGTGGTATCAACGCAGAGT-3′. RACE PCR products were cloned into the pCR4 Topo TA cloning vector, and plasmids from 10 colonies of each hemolymph 3′ RACE and cultured blood stage 5′ RACE were sequenced and analyzed for transcript start and stop points.

2.2. Quantification of transcript levels in cultured blood and hemolymph

Standard curves for *msa-1* and *bov57* were made by performing serial dilutions of known numbers of plasmid DNA (10^6 to 10^2 plasmid copy numbers). Each standard curve reaction and test sample RT+ and RT− cDNA (as a negative control for amplification) reactions were quantified in triplicate. DNA was isolated from a

100 μl aliquot of sample of either blood or hemolymph and run simultaneously in order to quantify total parasite numbers. A TaqMan assay with a PE Applied Biosystems (Carlsbad, California) fluorogenic probe was performed using primers amplifying msa-1 (forward, 5′-GATGCGTTTGCACATGCTAAG-3′; reverse, 5′-CGGGTACTTCGGTGCTCTCA-3′; probe sequence 5′-6FAMCACGCTCAAGTAGGAAATTTTGTTAAACCTGGATAMRA-3′) as previously described for the single copy *msa-1* gene (Howell et al., 2007b). *Bov57* primers (forward, 5′-GAAATGCGTGAGGACATCAACA-3′; reverse, 5′-CCACACGTAAACGCAATTGG-3′) amplified a 151 bp fragment between bp 1249 and 1399. The probe sequence 5′-6FAMTGCACAAAGGTCATGCTAAGGCTATTCTCTACGTAMRA-3′ annealed between bp 1307 and 1339 and the assay was performed under the same conditions previously described for *msa-1* (Howell et al., 2007b).

2.3. Expression of BOV57 in yeast

Recombinant protein was expressed in *Pichia pastoris* using the EasySelect *Pichia* Expression kit (Invitrogen). The full length *bov57* gene minus the predicted signal peptide (*mafaklsilftfllvhlvstna*) and stop codon was cloned into the pPICZα vector and transformed into TOP10 cells. The resulting construct contained a 5' vector sequence tag including a *c-myc* epitope used for detection of the recombinant protein in western blots by an anti-*c-myc* monoclonal antibody (Invitrogen). Plasmid DNA was isolated for transformation into the KM71H strain of *P. pastoris*. A polyhistidine tag was used for purification of the protein using the Pro-bond™ purification system (Invitrogen). Trypsin-digested recombinant protein was analyzed by liquid chromatography–tandem mass spectrometry to verify expression in the correct reading frame.

2.4. Monoclonal antibody production and protein expression

Monoclonal antibody production protocols were approved by the Washington State University Institutional Animal Care and Use Committee. One group of three female BALB/c mice was immunized subcutaneously with 10 μg of recombinant BOV57 subcutaneously in Freunds complete adjuvant. The mice were boosted twice subcutaneously at 14-day intervals with 10 μg of antigen in incomplete Freunds adjuvant. Following the third immunization, mice were bled from the lateral tail vein and sera screened using an indirect fluorescence assay (IFA). Briefly, slides were prepared from merozoite culture of known parasitemia or from infected hemolymph and frozen at −80 °C until use. Slides were fixed in acetone and mouse sera applied at a 1:100 dilution for 30 min. Following three PBS washes, an FITC-conjugated goat anti-mouse secondary antibody diluted 1:40 in 3% BSA in PBS was applied and allowed to incubate 30 min. Slides were washed again and observed under oil. Negative controls included normal mouse sera incubated with *B. bovis* merozoite slides and normal bovine erythrocyte slides incubated with recombinant BOV57 immunized mouse sera. The *msa-1* monoclonal antibody 23/10.36.18 (5 μg/ml) was used as a positive control.

Following initial IFA screening, the mouse producing sera with the strongest fluorescent reactivity to both merozoites and kinetes was selected for monoclonal production. Briefly, 72 h after receiving 10 μg antigen intravenously, the mouse was euthanized and splenic cells were harvested. Cell fusions and cloning were performed using previously described standardized protocols (Haldorson et al., 2006). Hybridoma supernatants were screened by IFA as described above and selected positive hybridomas cloned twice by limiting dilution and re-screened by IFA. Monoclonal antibody concentration and heavy and light chain isotypes were determined by ELISA as previously described (Haldorson et al., 2006).

Monoclonal 616 was used in IFA/DAPI to obtain images for publication. The IFA protocol was the same as above except that 250 mM NaCl PBS + 5% normal goat serum was used as a blocking buffer. A monoclonal against RAP-1 (23/53.156.77) was used as a positive control at 5 μg/ml per reaction. The reaction of blood smears with monoclonal antibody and DAPI (1 min incubation) were viewed and photographed using an Axio Imager.M1 microscope (Carl Zeiss Microimaging, Thornwood, NY) equipped with an LED illuminator for bright field microscopy and an X-Cite 120 Fl Illuminating system (EXFO Photonic Solutions, Mississauga, Ontario, Canada) for epi-fluorescence microscopy. For FITC, 490 nm excitation and 525 nm emission were used. For DAPI, 325 nm excitation and 425 nm emission were used. The microscope was equipped with an AxioCam MRc5 digital camera (Carl Zeiss Microimaging) connected to a computer workstation running AxioVision 4.5 imaging software (Carl Zeiss Microimaging). Pictures were taken at 40× magnification.

2.5. Sequencing of bov57 in geographically diverse strains of B. bovis

Bov57 was amplified from gDNA of Australian strains E61 and F100a, and Argentine strains L17v and L17a using forward primer 5′-ATGGCATTTGCAAAGTTGTCTATT-3′ and reverse primer 5′-TGAGGTTCGGTTGGATACGGACAT-3′. DNA was cloned into pCR4 Topo TA (Invitrogen) and miniprep DNA was sequenced. A total of 5 clones were sequenced from each strain. The resulting sequences were aligned using Vector NTI Suite 9.0.0 and translated polypeptides displayed using Boxshade 3.21 (http://www.ch.embnet.org/software/BOX_form.html).

2.6. Comparison of syntenic gene in B. bigemina and B. equi

Sequence data from *B. bigemina* was produced by the Pathogen Sequencing Unit at the Wellcome Trust Sanger Institute and was obtained from the *B. bigemina* genome project ftp site (ftp://ftp.sanger.ac.uk/pub/pathogens/Babesia/bigemina/). *B. equi* sequence data used in this study was obtained through the USDA/ARS *B. equi* genome sequencing project which can be accessed at http://genomics.vetmed.wsu.edu/index1.html.

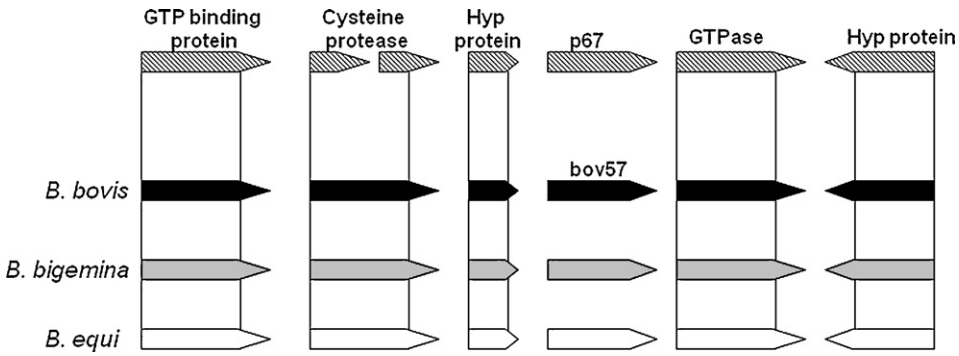

Fig. 1. Diagram of the conserved *Theileria parva* and *Theileria annulata* p67 locus (hatched) showing synteny among *Babesia bovis* (black), *Babesia bigemina* (grey), and *Babesia equi* (white). Genes clustered by sequence identity are indicated by connecting lines.

3. Results and discussion

Identification of syntenic gene blocks between the T2Bo strain of *B. bovis* and *T. parva* has been previously described (Brayton et al., 2007). The gene order of the p67/SPAG-1 locus in *T. parva* and *T. annulata* is: hypothetical protein, GTPase, p67/SPAG-1, hypothetical protein, 2 cysteine protease genes, GTP-binding protein (Fig. 1). In *B. bovis*, the gene order in this locus is conserved except there is only one cysteine protease gene present. Gene order is also conserved among two other *Babesia* pathogens of cattle and horses, *B. bigemina* and *B. equi* (Fig. 1). The percent amino acid similarity for *B. equi*, *B. bovis*, *T. parva*, and *T. annulata* proteins in this locus was determined through Clustal amino acid alignments. The percent similarity of the predicted amino acid sequence for the GTPase protein from *B. equi*, *B. bovis*, *T. parva*, and *T. annulata* was 69.6%. Similarities of the all of the genes in this locus were as follows: hypothetical protein (70%), GTPase (69.6%), hypothetical protein (83%), cysteine protease (72%), and GTP-binding protein (80.5%).

The gene occupying the position of *p67* in *B. bovis* (GenBank # BBOV_IV007750) was designated *bov57* based on the predicted Mw of its protein product (Mw = 57,584 Da), and was determined to be a single copy gene by performing a BLAST search against the completed T2Bo genome sequence. Unlike the gene encoding p67, which contains a 29 bp intron (Nene et al., 1992), there are no predicted introns in the gene encoding *bov57*. This was confirmed by sequence analysis of full length *bov57* cDNA, which matches the predicted reading frame in the genomic sequence. The p67 protein is predicted to have a transmembrane domain. The BOV57 protein contains a predicted N-terminal signal peptide but no C-terminal GPI anchor (the following online prediction programs were utilized: http://www.cbs.dtu.dk/services/TMHMM-2.0/ and http://mendel.imp.ac.at/sat/gpi/gpi_server.html).

When aligned to *T. parva p67*, the full length *B. bovis bov57* has 34.9% and 28.6% nucleotide sequence identity with *p67* and SPAG-1, respectively. At the amino acid level, there is 11.8% amino acid identity (19.6% similarity) between BOV57 and p67 and 6.7% amino acid identity (13.4% similarity) with SPAG-1. One possible hypothesis results from the sporozoite stage of *Theileria* invading a distinct host cell (lymphocyte) compared to the sporozoite and merozoite stages of *B. bovis* and *B. bigemina* (erythrocyte), therefore requiring different ligands. This hypothesis is supported by the observation that the *bov57* syntenic region of *B. bigemina* (a cattle pathogen) gene has higher sequence identity to *bov57* in *B. bovis* than in *B. equi* (a horse pathogen). Amino acid sequence identity between *bov57* and the corresponding predicted protein in *B. bigemina* and *B. equi* is 51% and 12%, respectively (Fig. 2). Although sequence identity is low, conserved residues are distributed across the length of the aligned polypeptides. It is possible that these conserved residues could be part of a conserved active site among these syntenic genes. Conservation among species of *Babesia* that infect different mammalian hosts and are transmitted by different life-cycle stages of *R. microplus* suggests that this protein may have an essential function in the life-cycle of these parasites.

When comparing transcript levels between cultured blood and tick stages, the single copy *msa-1* gene was chosen rather than a housekeeping gene because it encodes a surface protein that has been evaluated as a vaccine candidate (McElwain et al., 1998; Hines et al., 1995) and is transcribed in both merozoite and sporozoite stages. MSA-1 is postulated to be involved in host erythrocyte invasion and is known to contain epitopes that induce

Table 1
cDNSA and DNA copy number of *bov57* and *msa-1* in blood and hemolymph quantified by real-time PCR.

	Blood		Hemolymph	
	cDNA	DNA	cDNA	DNA
bov57	4.2×10^8	6.8×10^8	3.6×10^6	1.0×10^7
msa-1	4.2×10^9	6.6×10^8	3.6×10^4	$1/3 \times 10^7$
Difference in cDNA copy number	1000-fold *msa-1* > *bov57*		100-fold *bov57* > *msa-1*	

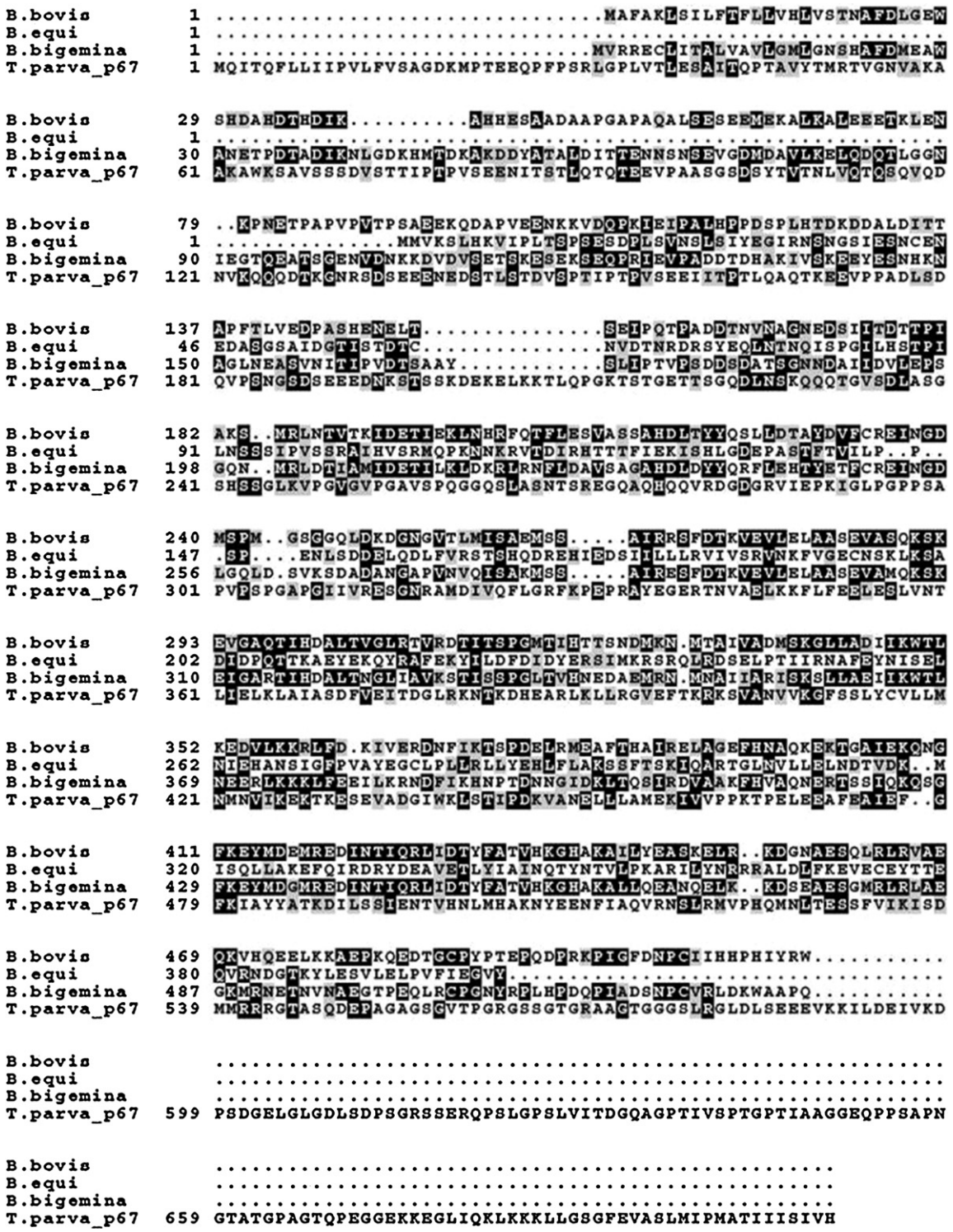

Fig. 2. Amino acid alignment of *p67* of *Theileria parva*, *bov57*, and syntenic genes of *Babesia equi* and *Babesia bigemina*.

production of sporozoite and merozoite neutralizing antibodies (LeRoith et al., 2006; Mosqueda et al., 2002). As expected when comparing two single copy genes, parasite numbers determined by real-time PCR from DNA isolated from both blood and hemolymph were consistent (Table 1). Overall, the *msa-1* gene was transcribed at higher levels (1000-fold) in merozoites isolated from culture. However, in infected hemolymph *bov57* was transcribed at a level 100-fold higher than *msa-1* (Table 1). Although, *bov57* transcription does not appear to be up-regulated in the two

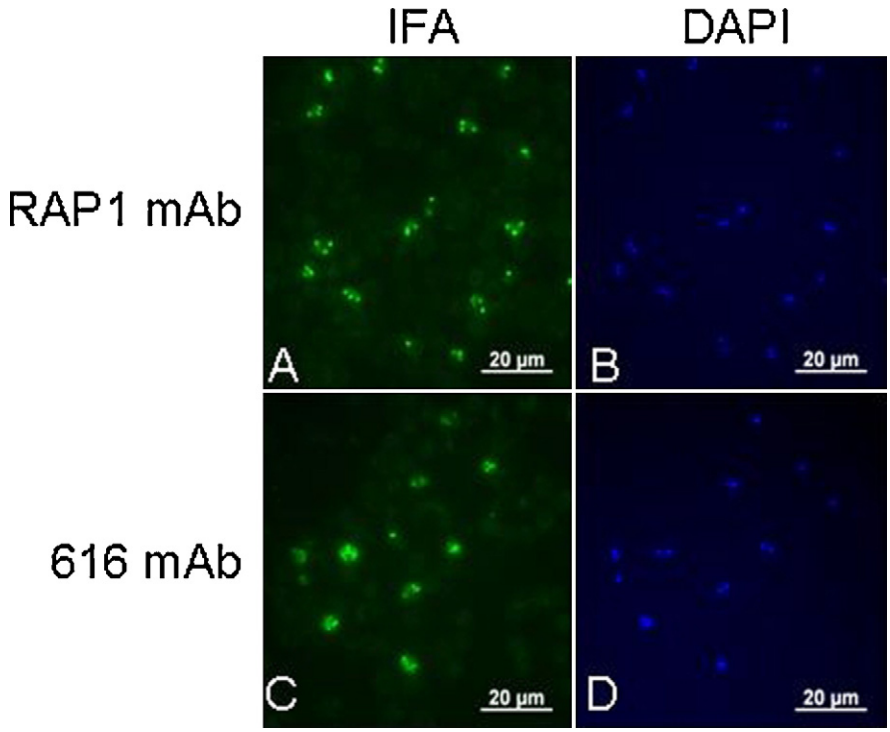

Fig. 3. Expression of BOV57 in *Babesia bovis* merozoites (T2Bo strain) as determined by IFA and DAPI. (A) IFA of a positive control RAP-1 monoclonal antibody 23/53.156.77; (B) RAP-1 monoclonal antibody following staining with DAPI; (C) IFA of monoclonal 616 against recombinant BOV57; (D) monoclonal 616 followed by staining with DAPI. Bar, 20 μm.

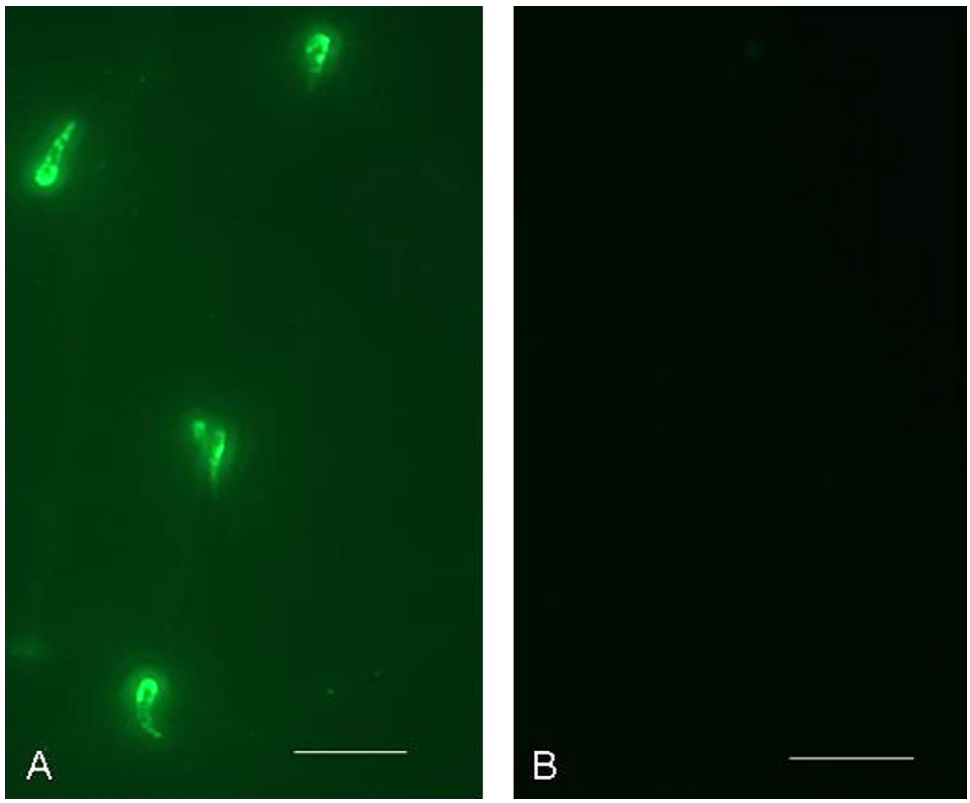

Fig. 4. Expression of *bov57* protein in kinetes of the T2Bo strain of *B. bovis* using IFA. (A) Monoclonal antibody 140/323.2.1 against recombinant BOV57; (B) negative control monoclonal antibody AnaF3216C1 against *Anaplasma marginale*. Bar, 20 μm.

stages that were tested, the increased *bov57* transcription levels in kinetes relative to a known surface protein encoding gene is interesting because it suggests that *bov57* could play a role in the biology of *B. bovis* within the tick. Using SMART RACE, we were able to confirm the 3′ and 5′ transcript ends of *bov57* in kinetes in the *R. microplus* kinete stage of the tick transmissible strain T2Bo. Using erythrocyte cultures of T2Bo and the biologically cloned Mo7 strain; we verified the 5′ transcript end of *bov57* in blood stages. RACE products were the same size as those predicted from the genome (data not shown).

Protein expression in merozoites (Fig. 3) and kinetes (Fig. 4) was verified using immunofluorescence. Two IgM monoclonals were produced, one with reactivity exclusively against cultured blood stages, and another with reactivity against the kinete stage. The fact that each monoclonal antibody reacted to only one stage (blood or tick) strongly suggests that different epitopes were targeted and were missing or masked perhaps by protein folding in the other stage. The punctate pattern of reactivity in cultured merozoites is distinctly different from the diffuse pattern seen with MSA-1, a known membrane protein. Demonstrating expression of BOV57 in both mammalian and vector stages represents a notable divergence from p67 of *Theileria*. Further studies will be performed to investigate expression of BOV57 in sporozoites.

To evaluate the level of sequence divergence among different strains of *B. bovis*, we sequenced the *bov57* gene in two Australian and two Argentine strains. BOV57 is highly conserved among these strains (97–100%) and is completely conserved between the attenuated and parental strains tested from each country. The positions of amino acid changes are also conserved and occur primarily at the N-and C-terminal ends. The high degree of BOV57 sequence conservation among strains including known immunogenic attenuated strains capable of inducing solid protection against virulent challenge (Shkap and Pipano, 2000), suggests that BOV57, if shown to be immunogenic in infected cattle, is a potential candidate component for a subunit vaccine.

In summary, *bov57* was identified through comparative genomic analysis of the p67 locus in *T. parva* with the conserved syntenic locus in *B. bovis*. The *bov57* gene is highly conserved among geographically diverse *B. bovis* strains, and if found to be immunogenic, could be a component of a broadly effective vaccine. *Bov57* is expressed in multiple life-cycle stages and may serve as an immune target in the mammalian host as well as a target for blocking transmission by the tick vector. While there is sequence divergence between *bov57* and *p67* that may have resulted from genetic drift, this does not exclude the possibility that these genes have similar functions. This locus is also conserved in *B. equi* and *B. bigemina*. The conservation of gene order within the loci we have described strongly indicates that these genes have related ancestry, and further investigation of *bov57* is warranted.

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